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Selective Laser Photocoagulation of Blood Vessels in a Hamster Skin Flap Model Using a Specific ICG Formulation

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Background and Objective: **The present study was undertaken to evaluate the selective laser photocoagulation of blood vessels in a hamster skin flap model using a specific indocyanine green (ICG) formulation.**
Study Design/Materials and Methods: **Experiments were performed in a hamster skin flap model after injection of ICG in aqueous solution (ICGA), or after injection of a specific formulation of ICG (ICG in emulsion: ICGE). Laser irradiation was achieved 30 minutes after injection with a 300 μm fiber connected to a 805 nm diode laser (power = 0.8W, spot diameter = 1.3 mm and pulse exposure time lasting from 1 to 5 s). Macroscopic observation and acute histology were performed to compare the tissue effects obtained for each ICG formulation and to assess the selectivity of vessel damage.**
Results: **The ICGE clearance process was slowed down as compared to the ICGA process. After 30 minutes, the concentration of ICG in blood is higher (2.27 ± 0.4, $P < 0.003$) for ICGE compared to ICGA. With ICGA, vessel coagulation required a minimum fluence of 240 J/cm^2, which led to very significant skin damage. Conversely with ICGE, vessel coagulation required a fluence of 120 J/cm^2. With such a fluence, no laser effect could be detected on the skin. Histological examination confirmed blood vessels coagulation in depth, whereas epidermis and dermis remained intact.**
Conclusion: **The major restrictions of ICG in aqueous solution, which are the very-short half-life of ICG in blood and consequently the lack of selectivity in blood vessels after a few minutes, are alleviated when ICG is used in emulsion. ICG in emulsion increases the circulating half-life of ICG and moreover confines ICG in the vascular compartment. Thanks to this specific property, it is possible to obtain a selective vascular damage 30 minutes after injection. Lasers Surg. Med. 21:365–373, 1997.**

Key words: 805 nm diode laser; blood vessel; skin; ICG; emulsion

INTRODUCTION: NEW APPROACH TO TREATING BLOOD VESSEL DAMAGE

Selective destruction of blood vessels is an important application of lasers, particularly in dermatology. The ability to close deep vessels

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would provide an important tool for the treatment of angiodysplasias of the skin, or superficial varicosities of the extremities. Occlusion of large vessels would allow treatment of tumors as well. In dermatology, although damaging blood vessels by heating hemoglobin with a yellow laser beam is relatively successful for the treatment of vascular lesions, two factors limit this technique: (1) specificity for thermal damage is limited by the absorption of light by melanin, and (2) an insufficient depth of light penetration prevents thick lesions from being successfully treated.

Recently, a new approach has been proposed, which requires a laser that is not absorbed by the biological tissue, but by a dye specifically located in the blood vessel. It has been shown that the 694 nm ruby laser irradiation after the Prussian blue injection can induce deeper vascular injury than the conventional pulse dye laser [1]. In addition, ICG dye-enhanced diode laser photocoagulation could achieve more selective treatment of blood vessels while producing less damage of adjacent tissular structures in skin or in tumor [2,3].

The principle is based on the lack of absorption sites for red (694 nm) or near-infrared (805 nm) radiations within biological tissues. Since the emission of the ruby laser corresponds to the absorption peak of the Prussian blue, or since the emission of the 805 nm diode laser corresponds to the absorption peak of ICG, dye-enhanced laser may allow selective destruction of dye-containing vessels.

Bass [4] has reported the treatment of several patients with angiodysplasias using systemic ICG injection or intralesional ICG injection and 805 diode laser irradiation. His results were encouraging, but the impossibility of assessing in real time the selectivity of blood vessel thermal damage was a major limitation. This impossibility was mainly due to the short half-life of ICG leading to a lack of selectivity after a few minutes. Reichel [5], using a similar technique, also has concluded that the laser treatment had to be performed almost immediately after intravenous administration of ICG and had to be completed within 2–3 minutes after initiation of photocoagulation. In addition, Ho [6] suggested that the very-short half-life of ICG in blood and the lack of selectivity in blood vessels could be a relative limitation for ICG dye-enhanced laser photocoagulation. Different techniques have been proposed to alleviate this restriction. Obana [7] has proposed an administration method that maintained a high steady-state plasma concentration of ICG. However, the leakage of dye from the vessels increases with the duration of the treatment. This phenomenon reduces the blood vessel selectivity. Libutti [8] also has proposed means to overcome this obstacle: (1) development of ligands that bind the dye particles to the intimal surface of vessels and increase the local chromophore concentration, and (2) direct distal intravenous or proximal arterial infusion using proximal application of a tourniquet to prevent rapid dye clearance (only feasible in case of superficial varicosities of the extremities).

We have demonstrated previously that ICG could be incorporated in emulsion [9]. This new approach may be an alternative way to increase the circulating half-life of ICG. Moreover, the incorporation of ICG in emulsion may confine ICG in the vascular compartment and limit the interaction of ICG with blood components. Emulsion formulations (water and oil: o/w) have been proposed to obtain long circulating systems for highly lipophilic drugs [10]. The hamster skin flap model has been proposed since it is usually considered to be a good correlate with those in cutaneous vascular malformations. In the hamster skin flap model, the vessel size ranges from 50 μm to 250 μm and the overall thickness of the preparation is approximately 1 mm. In cutaneous vascular malformations, several histological studies have shown that the size of the vessels can vary from 50 μm to 400 μm (mean size: 250 μm) with a distribution of these within the superficial and deep dermis [11]. A study carried out after excision of 26 angiomas of the face showed that 13 angiomas presented relatively superficial vessels (between 0.1 and 0.5 mm) in depth, whereas the other half comprised vessels affecting the totality of the dermis and even located uniquely at some depth (between 0.5 mm and 1 mm) [12].

The present study was undertaken to compare the selective destruction of blood vessels in a hamster skin flap model using a 805 nm diode after injection of a ICG in aqueous solution and a specific ICG emulsion formulation.

MATERIALS AND METHODS

Hamster Skin-flap Window Model

The hamster skin-flap window model is a chronic preparation of the skin and provides access from the exterior and interior surfaces. All the layers are intact, from the stratum corneum, composed of keratinocytes, stratum granulosum

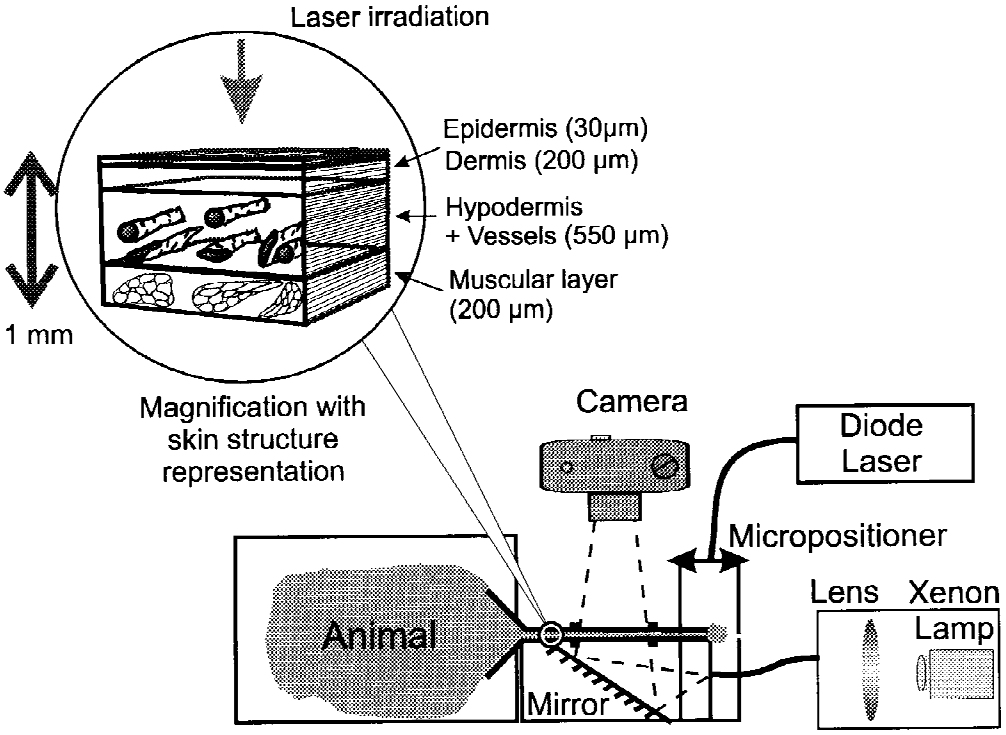

Fig. 1. Plan view of the experimental arrangement used to irradiate and to observe the skin-flap window.

between the stratum corneum and the basal cells and the fascial layers of the dermis. Arterioles, with diameters up to 150 μm and venules with diameters up to 250 μm, course on the fascial layers. The arterioles have circular sections, whereas the venules can have circular or ellipsoid. The larger venules are usually ellipsoid with the major diameter parallel to the windows. The overall thickness of the preparation is ~1 mm (epidermis: 30 μm, dermis: 200 μm, hypodermis: 550 μm, muscular layer: 200 μm) (Fig. 1). In the epidermis there are numerous hair follicles (the hair has been shaved and remaining roots have been removed with hair depilatory cream). The color of the preparation is light yellow pink with no pigmentation in most animals. In the lower back, there are two dark pigmented flank glands (males only), but these are always kept outside the window.

The window consists of two identical and symmetric aluminium plates. This model is based on one developed previously by Papenfuss et al. [13] and slightly modified by Gourgouliatos [14]. Each plate has a 18 mm central viewing aperture, and they are attached together surgically to provide support for the flap. The weight of each plate is 1.7 g. The implantation procedure is conducted under deep anesthesia and sterile conditions. Suture holes are used to secure the skin flap to the plates. The main steps are: (1) shaving the hair off the back of the hamster, (2) lifting the dorsal skin flap and securing it with sutures at the top of the plate, (3) removing one complete layer of skin and the underlying fascial and avascular tissue on the opposing side of the flap corresponding to an area equivalent to the window aperture, and (4) placement of the second plate over the prepared skin and connecting and securing it to the first plate with additional sutures. Male hamsters are used since they have less fat under the skin, making the implantation procedure easier and giving a better preparation for viewing. This model is chosen for these experiments since it is possible to distinguish two different targets: skin and blood vessels. Arterioles or venules are the main targets. Concerning the skin itself, small capillaries are effectively present, but their size and their numbers are too limited to give an effective conversion of light into heat since the amount of ICG is limited by the volume of these capillaries [15].

Indocyanine Green Dye (ICG)

ICG (Infracyanine®, Serb, Paris-France) was used. The absorption peak of ICG in plasma or

blood is ~805 nm. After a bolus injection, the ICG plasma concentration decay is usually described as a biexponential decay. Ott et al. [14] have defined ICG concentration in plasma as follows: $[ICG]_{plasma}(t) = -Aexp(-\alpha t) + Bexp(-\beta t)$, with $\alpha = 0.189 \pm 0.021$ min^{-1} and $\beta = 0.0356 \pm 0.0061$ min^{-1}. This α coefficient (0.189 min^{-1}) is in accordance with the data of literature about ICG half-life in plasma (3–5 minutes) [2,17]. Two different ICG formulations were used. First, an aqueous solution of ICG was reconstituted with sterile water immediately prior use (ICGA). Second, a formulation of ICG in emulsion was elaborated (ICGE). The formulation of ICGE was performed since small particles using phospholipids and soybean oil had been used as a long circulating emulsion system and it had been demonstrated that in this case the dye followed in part the pharmacokinetic of the particle and not only its own [18]. The emulsion was prepared with phosphatidylcholine (Egg, 100% purity, Lipoid K.G., Ludwigshafen, Germany) and soybean oil (Sigma Chemical, St Louis, MO) by extrusion through polycarbonates filters. The content of the emulsion formulation was adjusted to obtain 5% soybean oil, 1.75% glycerol w/v final volume, and 0.6% w/v phospholipid final concentration. After addition of the chloroformic phospholipid solution to the soybean oil and mixing, this solution was evaporated under N2 stream at room temperature. Addition of glycerol and water was performed just before ICG addition. Emulsification was carried out by extrusion through polycarbonates filters (Nuclepore, 0.2 μm diameter) 20 times. It must be noted that the preparation of ICGE required careful handling of ICG. The stability of ICG varied with concentration, light exposure, and high energy delivery systems such as sonication. The best procedure was to include dye just before the emulsification process. Spectral analysis have shown that ICG interact with the interfacial layer of the emulsion leading to a shift of the maximum absorption wavelength similar to that observed in blood sample [9]. ICGA and ICGE were sampled in order to inject a bolus at a dose of 15 mg/kg.

Blood Clearance

For each measurement, 20 μl were collected with heparinized capillaries on venous blood. After careful homogenization and dilution with isotonic glucose solution, the samples were centrifuged (10 min, 3,000 rpm/min). The supernatants were collected and blood samples fluorescence was measured with a spectrofluorometer (SHIMADZU RF-5000, Tokyo, Japan) in a 1 cm × 1 cm quartz cuvette. Before each clearance, a blank was realized with a blood sample before injection for each animal. Fluorescence intensity was measured at 760 nm excitation wavelength and 805 nm emission wavelength with half band widths of 10 nm.

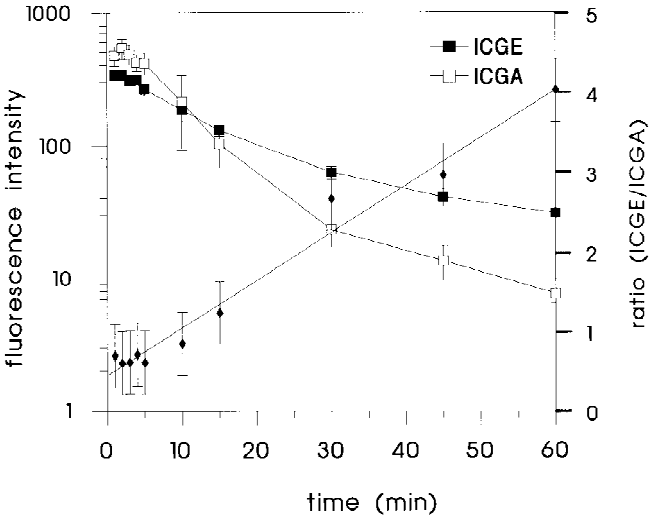

Fig. 2. Kinetic profiles of blood clearance measured by spectrofluorimetry (ICGA (□): ICG in aqueous solution, ICGE (■): ICG in emulsion). The fluorescence intensities are not statistically significant between 0–15 minutes. The statistically significant difference is first seen at 30 minutes ($P < 0.003$); $P < 0.003$ at 45 minutes, and $P < 0.001$ at 60 minutes (◆: ratio ICGE/ICGA).

Diode Laser

A 805 nm diode laser, model 2372-P3 (SDL, San Jose, CA) was used. The diode laser system was used in CW (continuous mode), noncontact mode (spot diameter = 1.3 mm), with a wavelength of 805 nm, a 0.8W power, and an incident power density of 60W/cm^2. The laser energy was delivered to the target tissue by an optic fiber with a core diameter of 300 μm and a numerical aperture of 0.37. During the experiments, the distance between tissue and the fiber was kept constant (3 mm) with a mechanical holder to give a 1.3 mm diameter spot (see Fig. 1). The laser beam profile and the beam diameter were controlled with a Beamscan (Photon, Los Gatos, CA). These parameters were used in order to compare the effects of laser irradiation with ICGA and ICGE.

Macroscopic Observation

The skin flap window was back-illuminated with a fiber bundle connected to a Xenon lamp.

TABLE 1. Tissue Response Observed Macroscopically, 1 Hour after Laser Irradiation*

Fluence (J/cm^2)	ICG in aqueous solution (ICGA)		ICG in emulsion (ICGE)	
	vessel response	skin response	vessel response	skin response
60 (n = 6)	no effect	skin dessication	vessel damage	no effect
120 (n = 8)	no effect	skin coagulation	selective vessel coagulation	no effect
180 (n = 8)	vessel damage	slight skin retraction	vessel coagulation + perivascular damage	no effect
240 (n = 8)	vessel coagulation	skin retraction	vessel coagulation	skin dessication
300 (n = 8)	vessel coagulation	important skin retraction	vessel coagulation	skin coagulation

*805 nm diode laser, power = 0.8 W, spot diameter = 1.3 mm, pulse exposure time lasting from 1–5 sec. Laser irradiations were performed 30–35 minutes after injection of a 1 ml bolus of ICG in aqueous solution (15 mg/kg) or ICG in emulsion (15 mg/kg).

Images were taken with a Nikon F301 camera connected to a 55 mm macro-lens (Nikon, Japan). Macroscopic observation was performed 2 hours after laser irradiation to quantify the damage. The position of each laser spot was located, its size was measured, and tissue modifications were observed. For blood vessels, thermal damage was graded as no effect, vessel damage, selective vessel coagulation, vessel coagulation. For the skin, thermal damage was graded as no effect, skin dessication, slight skin retraction, skin retraction, important skin retraction.

Histological Assessment

Animals were sacrificed 2 hours after laser exposure, immediately after macroscopic observation. The skin flap was dissected and immediately immersed in 4% buffered formaldehyde-0.2% picric acid for ~5 hours. The fragments of tissue, which included laser impacts, were dissected, isolated from surrounding tissue, and were routinely embedded in araldite. Semithin serial sections (1.5 μm thick) were cut and stained with trichrome solution. These sections were observed through an Axiophot microscope (CARL ZEISS, Oberkochen-Germany) at ×10, ×20 and ×40 objective magnifications. Histological study of several spots determined the thermal damage selectivity of laser impacts on the tissue.

MATERIALS AND METHODS

The first part of the study was focused on the ICGA and ICGE blood clearances. The blood clearance was studied into two groups of three animals.

The second part of the study focused on blood vessel photocoagulation selectivity when using ICGA and ICGE. The study was performed on six hamsters (3 hamsters with ICGA, 3 hamsters with ICGE). Under aseptic conditions, the hamsters were injected through the tongue vein with a bolus of ICGA, or with a bolus of ICGE at a dose of 15 mg/kg.

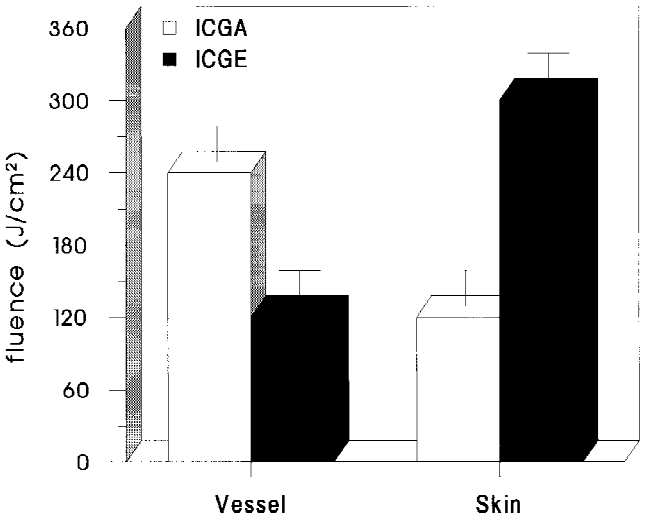

Fig. 3. Minimum fluence necessary for vessel and skin coagulation (ICGA: ICG in aqueous solution, ICGE emulsion: ICG in emulsion) observed 30 minutes after injection. Diode: 805 nm, spot diameter: 1.3 mm, pulse duration: 1–5 sec. (Error bars represent the standard deviation in the measurement of the fluence.)

For each hamster, the irradiance was 60W/cm^2. The laser energy was delivered in a single pulse mode. Single pulse exposure time was increased from 1–5 seconds. Fluences ranged between 60 J/cm^2 and 300 J/cm^2 (power = 0.8W, spot diameter = 1.3 mm for pulse exposure time lasting from 1 second to 5 seconds. Four to eight laser spots were performed on each skin-flap window (blood vessel or skin), 30–35 minutes after ICGA or ICGE injection. Macroscopic observations were performed 2 hours after laser irradiation in order to quantify the damage. Then animals were sacrificed and tissue samples, including the laser spots, were submitted to histological examination.

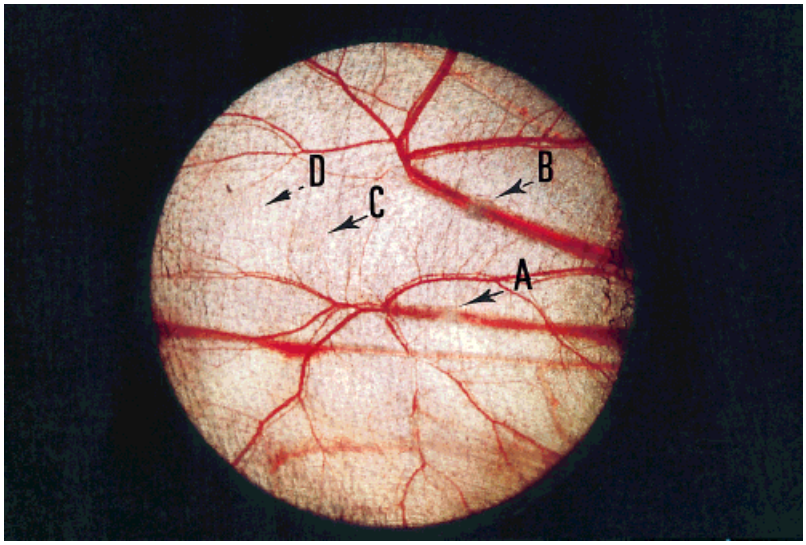

Figure 4

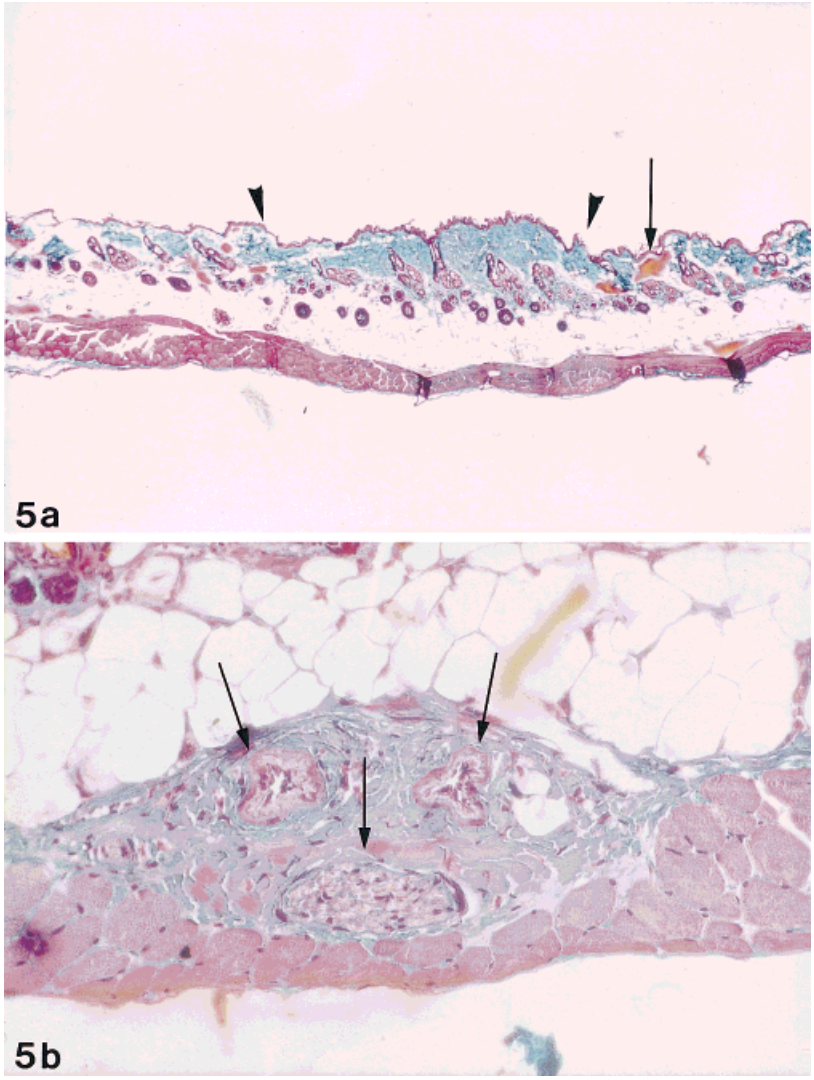

Figure 5

RESULTS

The kinetic profiles of blood clearance measured by spectrofluorometry for both ICGA and ICGE are shown in Figure 2. The clearance process is comparable between 0–15 minutes and the fluorescence intensities for ICGA and ICGE and consequently the concentrations of ICG in blood are not significantly different. Calculations give an ICGA half-life of ~5 minutes. The ratio (ICGE/ICGA) varies between 0.63 and 1.3 during this time period.

The second phase of clearance (15–60 minutes) shows that this process is slowed down for ICGE. Consequently, the concentration of ICG in blood is higher after ICGE injection when compared to that observed after ICGA injection. A statistically significant difference is first seen at 30 minutes ($P < 0.003$), and the ratio (ICGE/ICGA) reaches 2.27 ± 0.4. At 45 and 60 minutes, the ratio (ICGE/ICGA) is increased, respectively, to 3.0 and 4.07. However, the concentration of ICG in blood is reduced by a factor of 2 between 30 and 60 minutes.

The ratio (ICGE/ICGA) increases linearly as a function of time, and the following equation gives the relation between the ratio (ICGE/ICGA) and the delay after injection T(min):

$$\text{Ratio (ICGE/ICGA)} = 0.47 + 0.66\,[\text{T(min)}] \quad (r^2 = 0.98).$$

In order to evaluate the influence of the laser parameters chosen for this study, laser irradiations were performed without previous ICGA or ICGE injection, using fluences ranging from 60 J/cm^2 and 300 J/cm^2. In that case, there were no remarkable changes in the blood vessels or in the skin. Laser irradiations with the same parameters were performed 30–35 minutes after ICGA or ICGE injections. Macroscopic and microscopic observations allowed us to analyse 38 spots. Table 1 summarizes the effects obtained on the skin flap window. Figure 3 displays the minimum fluence required to obtain photocoagulation for each type of tissue and for each ICG formulation.

After ICGA injection, vessel coagulation required a minimum fluence of 240 J/cm^2. This fluence led to very significant skin damage. Skin retraction was always observed. Conversely, after ICGE, vessel coagulation required only a fluence of 120 J/cm^2. With such a fluence, no effect could be detected on the skin (see Fig. 5a). As illustrated by this figure, epidermis and dermis remained intact. In contrast, blood vessels located in the lower dermis were coagulated (see Fig. 5b). With ICGE, skin coagulation was observed only for fluences of 300 J/cm^2, sometimes with a slight skin retraction.

Figure 4 shows a skin-flap window 1 hour after laser irradiation and previous injection of ICG in emulsion. Note two examples of selective coagulation (spots A and B) of a blood vessel obtained, respectively, for fluences of 120 J/cm^2 and 180 J/cm^2. On this skin-flap window, two other laser irradiations were performed on the skin with similar parameters (spots C and D). No tissue changes can be detected.

DISCUSSION

This study allows the comparison of a new formulation of ICG using a long circulating emulsion carrier system (ICGE) versus ICG in aqueous solution (ICGA). ICG is a water-soluble tricarbocyanine dye that is mostly (90%) bound plasma proteins such as albumin, globulins, and lipoproteins. Only a few percent stays as a free dye. ICG bound to plasma proteins cannot leak out of the vessel and is eliminated by the enterohepatic route [17]. On the contrary, the free form can escape outside the blood vessel by crossing the vascular wall. Several studies have showed that extravasation is possible and that detectably amounts of ICG dye leave the vessels [19]. This was confirmed by ICG fluorescence imaging of the skin-flap window (data not shown). In case of ICGA, as previously described, plasma clearance is biphasic showing a rapid initial phase with a half-time of 3–5 minutes and a secondary phase with a half-time of >1 hour at very low concentrations not relevant for photocoagulation. This very rapid clearance is in accordance with our data

Fig. 4. Photograph of the window chamber after irradiation of blood vessels and skin 30 minutes after ICGE injection (diode: 805 nm, spot diameter: 1.3 mm) **A:** pulse duration: 2 sec: 120 J/cm^2; **B:** pulse duration: 3 sec: 180 J/cm^2. Note that with these parameters no effect is observed on skin (**C:** 120 J/cm^2 and **D:** 180 J/cm^2).

Fig. 5. Histological microphotographs showing the appearance of the skin after ICGE injection and laser irradiation 30 minutes later (diode: 805 nm, spot diameter: 1.3 mm, pulse duration: 2 sec, fluence: 120 J/cm^2). At low magnifications **(a),** no major effect is detected on epidermis and dermis (arrowheads indicate the limits of the injury; the arrow is pointed on eosinophilia on the edge of the spot). Within the hypodermis, higher magnification **(b)** reveals important lumen retraction of two arteries and thrombosis of the vein (arrows). Note the hyalinization of surrounding conjonctive tissue. Original magnifications, a: ×10; b: ×40.

presented in Figure 2 showing that the ICGA half-life is ~5 minutes.

For ICGE, the partition of ICG molecules is different. As emulsion particles enter the bloodstream, a different partition may occur and ICG can be: (1) bound to the plasma proteins, (2) in the free form, and (3) bound to emulsion particles. The mechanism of emulsion particles elimination is different from ICGA clearance. Emulsion particles are cleared by the reticulo-endothelial system like Kupffer cells in the liver [20]. This process is generally slower than the mechanism of ICGA elimination. This difference is clearly demonstrated in Figure 2 and is more pronounced during the second phase of clearance (30–60 minutes). After 30 minutes, the fluorometric study gives an absorption ratio of 2.27 ± 0.4 between ICGE and ICGA, which confirms that the ICGE elimination process is slow compared to ICGA. Similarly, the percentage of ICG in the free form should be affected. However, the fluorometric study does not allow to quantify directly the influence of emulsion particles on the partition of the free form.

In our laser experimental study, the laser irradiation is not performed immediately after dye injection, but 30 minutes after injection since this delay corresponds to the time for which a statistically significant difference is first seen between ICGE and ICGA, and the delay was rational in clinical practice. If a longer delay between injection and treatment leads to an increased ratio between ICGE and ICGA, respectively, 3.0 and 4.07 at 45 minutes and 60 minutes, it is associated with an important decrease of ICG in blood and consequently the need for a much higher power to obtain the same coagulation effect. Consequently, in our study, the coagulation is evaluated for ICGA and ICGE between 30–35 minutes after injection.

In the case of blood vessels, the fluence required to obtain blood vessel coagulation is 120 J/cm^2 for ICGE and reaches 240 J/cm^2 for ICGA. This is in accordance with the fluorometric study giving an absorption ratio of 2.27 ± 0.4 between ICGE and ICGA (Fig. 2). In the case of skin, the difference is even more pronounced. The dosage required for skin coagulation is 300 J/cm^2 for ICGE vs. 120 J/cm^2 for ICGA, giving a ratio equal to 2.5. This can be explained by a decrease of more than one-half of the free form crossing the vessel wall, due to the presence of the emulsion particles. Consequently when using ICGE, a better selective photocoagulation process is achieved due to a better confinement of ICG molecules inside the blood vessel and to a slowing down of the clearance process.

This microscopic study shows that it is possible to obtain a selective coagulation of blood vessels in depth (at least 1 mm) using ICG-enhanced 805 nm diode laser irradiation without any thermal damage to the epidermis and the dermis. Arteries present a complete shrinkage of their lumen; coagulation of veins is visualized by the lumen thrombosis. Some hyalinization of the conjonctive tissue also can been observed.

This is in accordance with previous observations demonstrating that red or near-infrared lasers induce a greater depth of vascular injury when combined with injection of a dye. When compared to the results obtained using wavelengths matching hemoglobin absorption, the difference is important. For example, Pickering [21] has assessed the maximum dermal depth where a vessel of a certain diameter can be selectively coagulated. He demonstrated that a 30-μm vessel could selectively be coagulated by a 577 nm pulsed dye laser only if its position in the dermis did not exceed 0.3 mm in depth. Similarly, Neumann [22] observed that the depth of vascular injury was limited to 0.44 mm with a Copper Vapor laser.

In conclusion, within the limits of the animal model used, our study demonstrated the feasibility of using ICG in emulsion in order to increase the circulating half-life of ICG and, moreover, to confine ICG in the vascular compartment. Thanks to these specific properties, it is possible to obtain a selective vascular damage 30 minutes after injection. Consequently, the major restrictions of ICG in aqueous solution, which are its very-short half-life of ICG in blood and its lack of selectivity in blood vessels after a few minutes, are alleviated when using ICG in emulsion.

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